



CONTRACT NO. A133-122  
FINAL REPORT  
APRIL 1994

**The Effects of Ozone Inhalation on  
Fibroblast Activation in the Lung:**  
***Possible Relationship to Long-Term  
Fibrotic Lung***

**CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY**



**AIR RESOURCES BOARD  
Research Division**



# The Effects of Ozone Inhalation on Fibroblast Activation in the Lung: Possible Relationship to Long-Term Fibrotic Lung

Final Report

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April, 1994



## ABSTRACT

The concern that environmental exposure to ozone may cause fibrotic remodeling of terminal airways is difficult to address experimentally in human volunteers because long term experimental exposures to ozone are difficult to carry out, and direct objective measures of lung fibrosis in living subjects are not currently possible. Despite these difficulties, it is possible to make indirect assessment of the potential for inhaled materials to provoke fibrosis in the lungs. One such method is to determine if airway and alveolar lining fluid collected at bronchoalveolar lavage (BAL) after exposure to ozone causes increased activity of cultured human lung fibroblasts (the specialized cells that are the exclusive source of collagen, and thus of fibrosis). Fibroblast activity can be measured by recording the extent of incorporation of radio-labelled thymidine - a component of cellular DNA. Thus, the principal goal of this work was to determine if brief exposure of human subjects to ozone in an environmental chamber results in increased fibroblast stimulating activity (FSA) of BAL. In addition, we measured transforming growth factors 1 & 2 (TGF $\beta$ 1 & TGF $\beta$ 2) in the BAL samples. TGF $\beta$ 1 and TGF $\beta$ 2 are proteins implicated as mediators of fibrosis in a variety of organ systems, including the lung. During two visits separated by 4 weeks, we exposed subjects to air and to ozone (0.4 ppm) for 3 hours while they performed intermittent moderate exercise. BAL was performed either within 1 hour (n=6) or between 16 and 18 hours of exposure (n=6). We found no significant difference in the FSA of the BAL or in the levels of TGF $\beta$ 1 in the BAL collected at the two time periods after ozone and air exposures. However, we found that the levels of TGF $\beta$ 2 were significantly higher in the BAL collected after the ozone exposures at both time points. Messenger RNA for TGF $\beta$ 1 and TGF $\beta$ 2 was found in the cell pellet of some but not all BAL samples, and ozone exposure did not influence the detection of these messenger RNAs. We conclude that although ozone exposure does not increase FSA in samples of airway and alveolar lining fluid, ozone exposure is associated with increased levels of TGF $\beta$ 2 - a cytokine implicated in the pathogenesis of lung fibrosis.

**ACKNOWLEDGMENTS**

The investigators wish to thank Jeff Golden, MD for his helpful advice about the application of fibroblast stimulating activity measurements in bronchoalveolar lavage and for assisting with some of the bronchoscopies. In addition, the investigators are grateful to Hofer Wong, BS, to Jane Liu, BS, and to Steve Edenson, BS for their excellent technical assistance in the conduct of this study.

**DISCLAIMER**

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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## SUMMARY

The project completed under this contract permits the following conclusions:

- (1) Fibroblast stimulating activity of bronchoalveolar lavage fluid is not increased at 1 or at 16-18 hours following exposure to 0.4 ppm ozone for 3 hours.
- (2) Ozone exposure is associated with increased levels of transforming growth factor  $\beta$ 2 (an inflammatory protein associated with lung fibrosis) but not with increased levels of transforming growth factor  $\beta$ 1 in bronchoalveolar lavage fluid.

## CONCLUSIONS AND SIGNIFICANCE

Our finding that brief exposure to a relatively high concentration of ozone does not result in increased fibroblast stimulating activity of bronchoalveolar lavage suggests that factors that activate fibroblasts and promote deposition of collagen in the lung may not be secreted into the air spaces and airways of the lungs during ozone exposures. However, our study design does not allow us to conclude definitively that environmental ozone exposure is not associated with fibroblast activation in the lung. For example, it is possible that the more frequent and sometimes more prolonged exposures to ozone encountered in real life may indeed result in increased secretion of fibroblast stimulating factors in the lung. In addition, it is possible that our in vitro technique for measuring the fibroblast stimulating activity of bronchoalveolar lavage is too insensitive to measure small changes that may have clinical significance. Finally, the fibroblast resides in parts of the lung removed from the airway epithelial surface



and the air-liquid interface of the alveoli that are sampled by the technique of bronchoalveolar lavage. Thus, ozone exposure may result in increased fibroblast stimulating activity in the tissues directly adjacent to fibroblasts but this increased activity would not be reflected in measurements made in bronchoalveolar lavage fluid.

Transforming growth factor  $\beta 2$  is a fibroblast growth regulatory factor, and its increased levels in bronchoalveolar lavage collected after ozone exposure suggest that ozone exposure may indeed promote secretion of certain pro-fibrotic growth factors. Further studies are needed to determine whether the immunoreactive TGF $\beta 2$  detected after ozone exposure is biologically active in causing fibroblast activation. In addition, further studies will be needed to determine the significance of our discordant findings of an increase in lavage TGF $\beta 2$  levels after ozone exposure but no significant change in TGF $\beta 1$  levels. Although we are unaware of previously published reports of functional differences between these structurally similar cytokines, our data suggests a differential effect of ozone on the activation and secretion of TGF $\beta 1$  and TGF $\beta 2$  in the lung.

Our findings are not directly comparable with other clinical data of ozone-induced lung fibrosis because, to our knowledge, there have been no previously published clinical studies of the effects of ozone exposure on lung fibrosis, fibroblast activation, or on levels of TGF $\beta 1$  or TGF $\beta 2$  in airway lavage fluid.

## RECOMMENDATIONS

We were unable to demonstrate differences in the activity of cultured human fibroblasts when we incubated them with bronchoalveolar lavage collected after air and ozone exposure, but our study necessarily had methodological limitations which preclude our dismissing the possibility of a link between ozone exposure and fibrotic remodeling of terminal airways. Newer techniques, as they become available, should be applied to study the possibility of a link between ozone exposure and fibroblast activation in the lung. In addition, because we found higher levels of the fibroblast regulatory protein TGF $\beta$ 2, there should be continued investigation of possible links between ozone exposure and increased pulmonary levels of other regulatory proteins such as other members of the TGF $\beta$  family, the A- and B- chains of platelet-derived growth factor, epidermal growth factor, insulin like growth factor-1, acidic and basic fibroblast growth factors, and fibroblast activating factor.

## INTRODUCTION

Animal experiments have revealed that long term exposure to ozone results in biochemical and histologic evidence of pulmonary fibrosis, especially fibrotic remodeling of terminal airways (1-4). Because it is not possible to conduct chronic ozone exposure studies or to sample lung tissue in human volunteers, it has not been possible to directly extend observations in animals on ozone-induced lung fibrosis to humans. However, epidemiological studies suggest that pulmonary function may be worse in residents of communities with high levels of photochemical pollution than in communities with low levels (5). In addition, longitudinal follow-up of pulmonary function in these residents revealed more rapid deterioration in lung function in residents of the more polluted areas. The lung function abnormalities were especially marked for those tests that reflect function of the small airways, the consistent site of damage in animal exposure studies.

The consistency of the association between chronic ozone exposure and collagen deposition in the lungs of animals indicate that ozone may directly or indirectly stimulate activation of lung fibroblasts, the unique source of collagen in the lungs. Measurement of thymidine incorporation into fibroblasts allows quantitative estimation of the degree of mitogenic activity of lung fibroblasts in culture such that the "fibroblast stimulating activity" of bronchoalveolar lavage fluid can be determined. For example, the fibroblast stimulating activity of bronchoalveolar lavage fluid from patients with fibrotic lung disease is increased compared to normal and decreases following corticosteroid treatment (6).

The studies in animals which have demonstrated that ozone exposure results in deposition of collagen in the lung have not investigated the mechanism by which this occurs. One possibility is that ozone inhalation evokes the generation of soluble growth

factors that initiate or modulate the proliferation of fibroblasts normally involved in wound healing and fibrosis (7-9). For example, activated macrophages express genetic messages and generate and release a variety of different growth factors that are mitogenic and stimulating for fibroblasts (10,11). One of the most important of these is transforming growth factor  $\beta$  (TGF $\beta$ ) (12-20).

In this study we asked two questions. The first question was whether brief exposure to ozone (0.4 ppm for 3 hours) increases the fibroblast stimulating activity in bronchoalveolar lavage from healthy subjects. The second was whether brief exposure to ozone results in increased levels of TGF $\beta$ 1 or TGF $\beta$ 2 in bronchoalveolar lavage and, if it does, whether the cellular source of these increased levels might be the pulmonary cells recovered in the lavage.

## **MATERIALS AND METHODS**

### Subjects

Nine healthy non-smoking subjects (age range 23 -36 years; 5 females) with no prior history of lung disease participated. All subjects signed consent forms approved by the Committee on Human Research at the University of California, San Francisco.

### Protocol

Bronchoalveolar lavage was collected and analyzed for fibroblast stimulating activity and for TGF $\beta$ 2 protein levels and for the presence of TGF $\beta$ 2 messenger RNA. BAL samples were obtained at two times: either within 1 hour (protocol I, n=6) or between 16 and 18 hours (protocol II, n=6) following 3 hour exposures to ozone (0.4 ppm). Control exposures to filtered air were also performed in each subject. The type of exposure was blinded to the subject and the order of first exposure was chosen randomly. Pulmonary function (spirometry and airway resistance measured by body plethysmography) was monitored before and after each exposure. Three of the subjects who participated in protocol I also participated in protocol II.

### Exposures

Subjects performed intermittent exercise (15 of each 30 minutes) on a cycle ergometer for 3 hours while wearing noseclips in a stainless steel and glass chamber (8X8X8 ft). The work loads were 80 watts for women and 100 watts for men. Ozone was generated by passing 100% oxygen through an ozonator (Wellsbach no. T-408) and concentrations were maintained at approximately 0.4 ppm as measured by an ultraviolet ozone analyzer (Dasibi no.1003 AH). Air was passed through a filter (type 91184, Mine Safety Appliances), and chamber temperature and relative humidity were monitored. The mean ( $\pm$ standard deviation) concentration of ozone during the filtered air exposures was  $0.095 \pm 0.001$  ppm and during the ozone exposures was

0.394±0.030 ppm. The mean temperature and relative humidities in the chamber during the filtered air and ozone exposures were similar (20.2 Vs 20.6°C; 42.5 Vs 45.7 RH, respectively).

### Pulmonary Function Tests

Spirometry was performed using a rolling seal Ohio 840 spirometer according to ATS criteria. Flow volume loops were generated by acquiring and digitizing the electrical volume signal versus time produced by the spirometer and then scaling these digitized computer counts to liters by application of a calibration factor. Flow was derived from the change in volume over the change in time (in real time) and flow versus volume was displayed on a graphic cathode ray screen as well as printed on hardcopy. All spirometric values were calculated from the raw data i.e volume versus time and the values reported are the best of three separate maneuvers.

Specific airway resistance was calculated for each subject 5 minutes after each dose of methacholine by measuring airway resistance and thoracic gas volume (Vtg) in a constant-volume variable pressure whole-body plethysmograph (Warren E. Collins Inc., Braintree, MA) every 30 seconds for 2.5 minutes.

### Bronchoscopy

Prior to bronchoscopy subjects were premedicated with atropine (0.6-1.0 mg intramuscularly) and then asked to gargle with dyclone 0.5%. Lidocaine 4% was sprayed to the posterior pharynx and then cotton swabs dipped in 4% lidocaine were placed in the pyriform sinuses to block the superior laryngeal nerves. Finally, 2.5 mls of lidocaine 1% was dripped along the wall of the throat using a laryngeal canula. The bronchoscope (Pentax FB-19D, Pentax Precision Instrument Corporation, Orangeburg, New York) was introduced orally and cough was further suppressed by frequent bronchoscope-delivered aliquots of 1% lidocaine (2 mL) so as to allow

insertion of the bronchoscope tip in a subsegmental bronchus of either the right middle lobe or lingula. Bronchoalveolar lavage (BAL) was performed by instilling four 60 mL boluses of sterile saline solution (warmed to 37°C), and suctioning immediately after each instillation. The lavage return was immediately pooled and mixed without gauze filtration.

#### Cell Count and Differential

Ten microliters of the pooled BAL was used to determine the total cell count of the BAL samples using a standard hemacytometer, and 250 $\mu$ L aliquots were spun in a cytocentrifuge (model 7 cytospin; Shandon Scientific, Sewickley, PA) onto glass slides that were then stained using the May Grunwald Giemsa stain. Two investigators, blinded to the subject's exposure history, each counted at least 200 cells and the cell counts of both investigators were averaged to yield the final percentages reported here.

#### Measurement of Fibroblast Stimulating Activity

Bronchoalveolar lavage cells were separated from the lavage fluid by centrifugation at 2000 rpm for 5 minutes at room temperature. The mitogenic effect of the bronchial lavage supernatant was measured by its ability to stimulate the uptake of 3H thymidine in human lung fibroblasts obtained from a lung biopsy. The fibroblasts were grown in DME H-21 FBS (UCSF cell culture), and when confluent were trypsinized and washed 3 times with media containing 0.5% FBS. These cells were plated in a 96 well plate (Corning) (5000 cells in 160 $\mu$ L), starved for 4 days in the low serum media, and then treated with 40 $\mu$ L of the supernatant obtained from the lavage samples. After 24 hours 20  $\mu$ L of 25  $\mu$ Ci/mL 3H thymidine (ICN) was added and the cells were incubated overnight. After detachment from the wells using trypsin, the cells were deposited on glass fiber filters using a cell harvester. These filters were then counted in a scintillation counter to determine thymidine incorporation.

#### Measurement of Messenger RNA for TGF $\beta$ 1 and TGF $\beta$ 2

The bronchoalveolar lavage cell pellet was resuspended in 4M GIT with 25 mM betamercaptoethanol. The RNA was then extracted by the phenol/chloroform method. The TGF $\beta$ 1 and TGF $\beta$ 2 cDNAs were prepared using M-MLV reverse transcriptase (Promega Corp) and oligo dT 2.5 ng/mL (Promega Corp). The reaction was performed at 37°C for 1 hour. Twenty percent of the CDNA from the reverse transcriptase reaction was used in the polymerase chain reaction which was performed with 0.025u/ $\mu$ L of Taq polymerase (promega), 0.1 M primers and 0.2 mM DNTPs. A cycle consisted of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes. After 35 cycles and a final 8 minute extension at 72°C, 10% of the PCR products were loaded onto a 3% agarose gel for electrophoresis. The PCR products were then visualized with EtBr.

#### Measurement of TGF $\beta$ 1 protein levels in bronchoalveolar lavage

TGF $\beta$ 1 levels were assayed in concentrated lavage samples using a commercially available enzyme-linked immunosorbent assay (Genzyme Diagnostics, Cambridge, MA). Three or four milliliter aliquots of bronchoalveolar lavage supernatant were first concentrated by dialysis twice against ammonium bicarbonate (50 mM, pH 8.0), and then overnight lyophilization. Lyophilized samples were resuspended in normal saline to a total volume of 300  $\mu$ l to yield lavage samples that were concentrated an average of 20-fold. These concentrated samples were sequentially acidified by addition of hydrochloric acid (1N) for 60 minutes, neutralized by addition of NaOH, and finally assayed for TGF $\beta$ 1 in accordance with the assay protocol.

#### Measurement of TGF $\beta$ 2 protein levels in bronchoalveolar lavage

Three milliliter aliquots of bronchoalveolar lavage supernatant were first acidified by addition of glacial acetic acid (M) to yield a molar concentration in the sample of 1 M



acetic acid. The samples were then dialyzed twice against ammonium bicarbonate (50 mM, pH 8.0) and lyophilized overnight. The lyophilized lavage was resuspended in 300  $\mu$ L normal saline. This procedure resulted in the lavage supernatant samples that were concentrated an average of 15-fold. TGF $\beta$ 2 levels were assayed in the concentrated samples using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN)

### STATISTICS

Data was entered on a computer spreadsheet (Microsoft Excel<sup>®</sup>, Microsoft, Redmond, WA) and exported to a statistics program (Statview<sup>®</sup>, Abacus Concepts Inc., Berkeley, CA) for descriptive and comparative statistics. All of the data was normally distributed and so is presented as the mean and standard deviation of the mean (mean $\pm$ SD). The two tailed paired t test was used for all analyses and differences with a p value < 0.05 was accepted as statistically significant.

## RESULTS

Mild bronchoconstriction, as reflected by a rise in specific airway resistance and a fall in maximum expiratory flow, was evident immediately after ozone exposures but not after filtered air exposures and this resolved by 16 to 18 hours in the six subjects who had these later measurements made (Table 1). Total and differential cell analysis on the BAL samples collected after air and ozone exposures revealed significant increases in neutrophil cell counts and in the percentage of recovered cells that were neutrophils (Table 2). These increases were larger in the BAL samples collected 16-18 hours after ozone exposure.

The mean fibroblast stimulating activity of the BAL was not significantly different after air and ozone exposures (Table 3). In addition, there was no significant difference in the fibroblast stimulating activity of lavage collected 16 - 18 hours after ozone exposure compared to the lavage collected within 1 hour of ozone exposure.

TGF $\beta$ 2 levels in the BAL collected after ozone were significantly higher than after air exposures when the lavage was collected either within 1 hour or between 16 and 18 hours after ozone exposure (Table 4). The size of the increase in the TGF $\beta$ 2 in the lavage samples was similar whether the post-exposure lavage was collected at 1 hour or at 16-18 hours. In contrast, the levels of TGF $\beta$ 1 were not significantly different after air and ozone exposures (Table 5). However, TGF $\beta$ 1 concentrations in BAL collected after air exposures were significantly higher than the paired concentrations of TGF $\beta$ 2 in the same BAL samples (Tables 4 and 5)( $p < 0.05$ ).

Messenger RNA for TGF $\beta$ 1 and TGF $\beta$ 2 was only detectable in a minority of the cell pellets from the BAL samples (Table 6). Exposure to air or to ozone did not influence the detection of message for either of these two proteins.

## DISCUSSION

The main findings of our study is that the fibroblast stimulating activity of bronchoalveolar lavage fluid is not increased significantly following exposure to 0.4 ppm ozone for 3 hours, but that ozone exposure is associated with increased levels of transforming growth factor  $\beta$ 2 in bronchoalveolar lavage both at 1 and 16-18 hours post exposure. Our results show that although ozone exposure does not increase fibroblast stimulating activity in samples of airway and alveolar lining fluid, ozone exposure is associated with increased levels of TGF $\beta$ 2 - a cytokine implicated in the pathogenesis of disorders associated with lung fibrosis.

Our finding that brief exposure to a relatively high concentration of ozone does not result in increased fibroblast stimulating activity of bronchoalveolar lavage is somewhat reassuring for it provides indirect evidence that activation of fibroblasts is not a composite effect of the multiple inflammatory mediators released into the air spaces and lumen of the lung during ozone exposure. However, our methods have sufficient limitations that this reassurance be tempered. For example, it is possible that the more frequent and sometimes more prolonged exposures to ozone encountered in real life may indeed result in net fibroblast stimulating activity in the lung. In addition, it is possible that our *in vitro* technique for measuring the fibroblast stimulating activity of bronchoalveolar lavage is too insensitive to measure small changes that may have clinical significance. Finally, the fibroblast resides in parts of the lung removed from the airway epithelial surface and the air-liquid interface of the alveoli, the areas of lung sampled by the technique of bronchoalveolar lavage. Thus, ozone exposure may result in increased fibroblast stimulating activity in the tissues directly adjacent to fibroblasts, but this increased activity will not be reflected in measurements made in bronchoalveolar lavage.

We found increased levels of TGF $\beta$ 2 but not of TGF $\beta$ 1 in bronchialveolar lavage samples collected at both early (within 1 hour) and late (16-18 hours) time points after ozone exposure. TGF $\beta$ 1 and 2 are members of the transforming growth factor- $\beta$  family of proteins that regulate cellular activities in the lung (19). TGF $\beta$  stimulates the synthesis of individual matrix proteins including fibronectin, collagens and proteoglycans and the potential for a pathologic role for TGF $\beta$  in diseases associated with organ fibrosis has been explored (14-17). For example, TGF $\beta$  may be involved in the pathogenesis of diabetic nephropathy (14), and TGF $\beta$  expression is elevated in the lungs of patients with idiopathic fibrosis (15). In addition, increased TGF $\beta$  gene expression is associated with bleomycin-induced pulmonary fibrosis (16). TGF $\beta$ 1 is the first discovered and prototypic member of this family and, although the product of a different gene, TGF $\beta$ 2 demonstrates over 70% similarity of the amino acid sequence in the C-terminal mature region (19). Experimental evidence to date show that TGF $\beta$ 1 and TGF $\beta$ 2 interact with the same cell surface binding molecules (19) and suggest that any differences in the functional effects of TGF $\beta$ 1 and TGF $\beta$ 2 on regulation of the extracellular matrix are quantitative rather than qualitative (20). In this context our finding of a differential effect of ozone exposure on TGF $\beta$ 1 and TGF $\beta$ 2 levels in BAL is surprising, for all of the above evidence would have predicted a similar effect of ozone exposure on both of these proteins. Our finding that ozone exposure results in hypersecretion of TGF $\beta$ 2 but not of TGF $\beta$ 1 in BAL suggests that ozone has different effects on the activation of secretion of these proteins in the lung, and that they may have different regulatory roles in the pulmonary response to ozone.

The finding of increased TGF $\beta$ 2 in the lavage collected after ozone exposure in the absence of increased FSA in the same samples can be explained by a number of possibilities. First, it is known that TGF $\beta$  has immunosuppressive activity and that it therefore does not always have pro-fibrotic activity (13, 19). It is best viewed as a

fibroblast regulatory protein, and its activation or inhibition of fibroblast collagen deposition may be a function of its concentration. Second, other inflammatory mediators released in the lung as a consequence of ozone exposure may serve to inhibit the profibrotic activity of TGF $\beta$ . In vitro, both  $\gamma$ IFN and TNF counteract the profibrotic activity of TGF $\beta$ . Thus, the levels of TGF $\beta$ 2 as well as the co-release of other inflammatory mediators provoked by the same stimulus may both be factors that influence the biologic effects of TGF $\beta$ 2.

Messenger RNA for both TGF $\beta$ 1 and TGF $\beta$ 2 was only found in a minority of the cell pellets from the BAL samples obtained after air and ozone exposures (table 5). The type of exposure or the time of collection of BAL did not influence whether message was detectable. The predominant cell in BAL is the macrophage so it is likely that the macrophage is the source of message in those samples where message was detected. However, because all BAL samples had both TGF $\beta$ 1 and TGF $\beta$ 2 protein detected, but all samples did not have detectable messenger RNA for TGF $\beta$ 1 or  $\beta$ 2 in the lavage pellet, other pulmonary cells, not present in lavage, must be the cellular source of these TGF $\beta$  proteins. Recent data show that bronchial epithelial cells are important sources of TGF $\beta$ 1 and  $\beta$ 2 (21), so that epithelial cells may well be important sources of the TGF $\beta$  protein detected in these BAL samples.

In summary, we found that ozone exposure is not associated with increased fibroblast stimulating activity in bronchoalveolar lavage fluid collected following exposure to 0.4 ppm ozone for 3 hours. However, we also found that ozone exposure is associated with increased levels of transforming growth factor  $\beta$ 2 but not of TGF $\beta$ 1 in bronchoalveolar lavage, findings which demonstrate that at least one cytokine with pro-fibrotic activity is released in the lung following ozone exposure.

## REFERENCES

1. Hussain MZ, Cross CE, Mustafa MG, Bhatnagar RS. Hydroxyproline contents and prolyl hydroxylase activities in lungs of rats exposed to low levels of ozone. *Life Sci* 1976;18:897.
2. Last JA, Greenberg DB. Ozone-induced alterations in collagen metabolism of rat lungs. *Toxicol Appl Pharmacol* 1980;55:108.
3. Last JA, Hesterberg TW, Reiser KM, Cross CE, Amis TC, Gunn C, Steffey EP, Grandy J, Henrickson R. Ozone-induced alterations in collagen metabolism of monkey lungs: Use of biopsy-obtained lung tissue. *Toxicol Appl Pharmacol* 1981;60:579.
4. Last JA, Reiser KM, Tyler WS, Rucker RB. Long-term consequences of exposure to ozone. *Toxicol Appl Pharmacol*. 1984;72:111.
5. Detels R, Tashkin DP, Sayre JW, Rokaw SN, Coulson AH, Massey FJ, Wegman DH. The UCLA population studies of chronic obstructive respiratory disease. *Chest* 1987;92:594.
6. Adolff CA, Golden JA, Gamsu G, Goetzyl EJ, Turck CW. Reduction in pulmonary fibroblast stimulating activity as an index of response to treatment of interstitial lung diseases. *New Eng J Med* 1990;322:1890.
7. Wahl SM, Wahl LM, McCarthy JB. Lymphocyte-mediated activation of fibroblast proliferation and collagen production. *J Immunol* 1978;121:942.
8. Banda MJ, Knighton DR, Hunt TK, Werb Z. Isolation of a nonangiogenesis factor from wound fluid. *Proc Natl Acad Sci USA* 1982;79:7773.
9. Turck CW, Dohlman JG, Goetzyl EJ. Immunological mediators of wound healing and fibrosis. *J Cell Physiol (Suppl)* 1987;5:89.
10. Schilling JA, Favata BU, Radokovich M. Studies of fibroplasia in wound healing. *Surg Gynecol Obstet* 1953;96:143.

11. Derynk R, Roberts AB, Winckler ME, Chen EY, Goeddel DV. Human transforming growth factor  $\alpha$  - Precursor structure and expression in E. Coli. *Cell* 1984 38:287.
12. Border WA, Ruoslahti E. Transforming growth factor- $\beta$  in disease: the dark side of tissue repair. *J Clin Invest* 1992;90:1.
13. Okuda SLR, Languino E, Ruoslahti E, Border W. Elevated expression of transforming growth factor  $\beta$ 1 and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. *J Clin Invest* 1990;86:453.
14. Broekelmann TJA, Limper AH, Colby TV, McDonald JA. Transforming growth factor  $\beta$  is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88:6642.
15. Wahl SM. Transforming growth factor beta (TGF- $\beta$ ) in inflammation: A cause and a cure. *J Clin Immunol* 1992;12:61.
16. Khalil N, Berezney O, Sporn M, Greenburg AH. Macrophage production of transforming growth factor  $\beta$  and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 1989;170:727.
17. Limper AH, Colby TV, Sanders MS, Asakura S, Roche PCC, DeRemee RA. Immunohistochemical localization of transforming growth factor  $\beta$ 1 in non-necrotizing granulomas of pulmonary sarcoidosis. *Am Rev Respir Dis* 1994:197.
18. Pelton RW, Johnson MD, Perkett EA, Gold LI, Moses HL. Expression of transforming growth factor- $\beta$ 1,  $\beta$ 2, and  $\beta$ 3 mRNA and protein in the murine lung. *Am J Respir Cell Mol Biol* 1991;5:522-530.
19. Pelton RW, Moses HL. The beta-type transforming growth factors. *Am Rev Respir Dis* 1990;142:S31-S35.
20. Magnan A, Frachon I, Rain B, Peuchmaur M, Monti G, Lenot B, Fattal M, Simmineau G, Galanaud P, Emilie D. Transforming growth factor  $\beta$  in normal human lung: preferential location in the bronchial epithelial cells. *Thorax* 1994;49:789-792.

20. Graycar JL, Miller DA, Arrick BA, Lyons RM, Moses HM, Derynck R. Human transforming factor  $\beta$ 3: recombinant expression, purification and biological activities in comparison with transforming growth factors- $\beta$ 1 and  $\beta$ 2. *Molecular Endocrinol* 1990;7:1977-1986.
21. Magnan A, Frachon I, Rain B, Peuchmaur M, Monti G, Lenot B, Fattal M, Simmineau G, Galanaud P, Emilie D. Transforming growth factor  $\beta$  in normal human lung: preferential location in the bronchial epithelial cells. *Thorax* 1994;49:789-792.



**TABLE 1****PULMONARY FUNCTION BEFORE AND AFTER AIR AND OZONE EXPOSURES**

Protocol I	AIR		OZONE		Protocol II		AIR		16-18h		Ozone		16-18 h	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
SRAW (L/cmH <sub>2</sub> O/L/s)	5.6±1.9	5.5±1.4	5.7±1.4	7.1±1.3*	5.5±1.0	5.7±1.2	6.0±0.9		5.9±1.7	7.6±2.2	6.0±1.6			
FEV1 (L)	3.8±0.3	3.8±0.3	3.7±0.4	3.3±0.4*	4.2±0.7	4.3±0.7	4.1±0.8		4.1±0.8	3.6±0.6*	4.0±0.8			
FVC (L)	4.4±0.5	4.4±0.6	4.4±0.6	4.0±0.5*	5.0±1.0	5.0±1.1	4.9±1.0		4.9±1.1	4.6±0.8	4.9±1.1			
FEF <sub>25-75</sub> (L/s)	4.3±0.8	4.4±0.9	4.2±0.8	3.6±0.7*	4.5±0.7	4.5±0.9	4.5±1.1		4.3±0.8	3.6±1.1*	4.3±0.8			

\*significantly different than the change after air exposure, p<0.05.

**Abbreviations:** SRaw denotes the specific airway resistance (the product of airway resistance and the lung volume at which the resistance measurement was made). FEV1 denote the forced expired volume in one second; FVC denotes the forced vital capacity; FEF 25-75 denotes the forced expired flow between 25 and 75% of the forced vital capacity.

**TABLE 2**  
**Percentage and Number of Neutrophils in Bronchial Wash and Bronchoalveolar Lavage  
 After Air and Ozone Exposures**

	1 Hour post Exposure		P VALUE	16-18 Hours Post Exposure		P VALUE
	AIR	OZONE		AIR	OZONE	
Neutrophil %	0.6±0.6	7.7±7.7	0.03	1.2±0.8	13.7±8.5	0.01
Neutrophil Cell Count (x10 <sup>4</sup> /mL)	0.1±0.1	2.0±2.1	0.006	0.4±0.3	3.4±2.6	0.05

**TABLE 3**  
**Fibroblast Stimulating Activity (CPM) of Bronchoalveolar Lavage**  
**After Air and Ozone Exposures**

Subject	1 Hour Post Exposure		Subject	16-18 Hours Post Exposure	
	Air	Ozone		Air	Ozone
1	1389	1054	1	1424	777
2	1214	703	2	1665	1401
3	543	675	3	989	961
4	659	911	7	669	709
5	451	1349	8	1374	1008
6	723	741	9	430	762
<b>Mean</b>	830	906		1092	936
<b>Std Dev</b>	381	261		479	257

**TABLE 4**  
**TGF $\beta$ 2 Levels (pg/mL) in Bronchoalveolar Lavage after Air**  
**and Ozone Exposures**

Subject	1 Hour Post Exposure		Subject	16-18 Hours Post Exposure	
	Air	Ozone		Air	Ozone
1	5.1	9.9	1	8.5	16.6
2	4.1	6.5	2	0.8	5.1
3	0.9	2.0	3	7.1	9.2
4	6.1	16.6	7	1.2	4.6
5	8.9	9.2	8	4.2	4.0
6	1.1	9.9	9	0.9	9.2
<b>Mean</b>	4.4	9.0*		3.8	8.1*
<b>S D</b>	3.1	4.8		3.4	4.7

\*Significantly greater than after air exposure,  $p < 0.05$ .

**TABLE 5**  
**TGF $\beta$ 1 Levels (pg/mL) in Bronchoalveolar Lavage after Air and Ozone Exposures**

Subject	1 Hour Post Exposure		Subject	16-18 Hours Post Exposure	
	Air	Ozone		Air	Ozone
1	28.2	27.5	1	16.5	28.8
2	31.2	20.5	2	40.6	18.5
3	10.7	5.0	3	3.5	12.6
4	5.3	5.3	7	8.6	18.1
5	14.6	3.7	8	13.3	11.9
6	27.5	14.1	9	26.6	35.8
<b>Mean</b>	19.6	12.7		18.1	20.9
<b>S D</b>	10.8	9.8		13.5	9.5

**TABLE 6**  
**Messenger RNA for TGF $\beta$ 1 and TGF $\beta$ 2 in the Cell Pellet of Bronchoalveolar Lavage After Air and Ozone Exposures**

1 Hour Post Exposure					16-18 Hours Post Exposure				
Subject	TGFβ1		TGFβ2		Subject	TGFβ1		TGFβ2	
	Air	Ozone	Air	Ozone		Air	Ozone	Air	Ozone
1	IS	IS	-	-	1	-	-	+	+
2	IS	IS	-	-	2	+	+	-	+
3	IS	IS	-	-	3	-	-	+	-
4	+	-	-	-	7	+	-	+	+
5	IS	IS	-	-	8	-	+	-	-
6	+	+	-	-	9	+	-	-	-

**Abbreviations:** IS denotes insufficient sample.



